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ZDZISŁAW TARGOŃSKI

N-ACETYLNEURAMINIC ACID AS A STANDARD IN DETERMINATION OF PECTATE LYASE ACTIVITY WITH THIOBARBITURIC ACID

Department of Chemistry and Food Technology, Agricultural University, Lublin

Key words: pectate lyase, N-acetylneuraminic acid, β-formyl-pyruvic acid, 4-deoxy-5-ketouronic acid.

A colorimetric method has been applied to determine the activity of pectate lyase in absolute units. The colour reaction was developed, using 2-thiobarbituric acid and oxidation product of N-acetylneuraminic acid and 4-deoxy-5-ketouranic acid.

The activity of pectate lyases is determined mainly by two methods. One method utilizes the change in light absorption of the mixture, measured at wavelength 235 mm as a result of pectin transelimination by pectate lyases and formation of unsaturated oligogalacturonic acids. The method is characterized by a low sensitivity which is expressed in a low value of the molar coefficient of absorption $E = 4600\ l \times mol^{-1} \times cm^{-1}$ (2).

The second method is based on the colour reaction of products of pectin transelimination mostly constituted by 4-deoxy-5-ketouronic acid and 4,5 unsaturated digalacturonic acid with 2-thiobarbituric acid [5, 6].

The method is highly sensitive, however the lack of readily accessible standard substances makes it impossible to express pectate lyases activity in absolute units and a preparative obtaining of one of the compounds resulting from pectin transelimination causes many difficulties.

The aim of the present work was to examine the possibilities of utilization of N-acetylneuraminic acid as a standard for plotting a standard curve in order to determine colorimetrically the compounds produced as a result of pectin degradation during colour reaction with 2-thiobarbituric acid. In the method of the experiments, the reaction of oxidation of products of pectin transelimination was utilized, i.e. of 4-deoxy-5-ketouronic acid and 4, 5 unsaturated digalactouronic acid and of N-acetylneuraminic acid with the use of periodate solution into β-formyl-pyruvic acid and colour reaction of this acid with 2-thiobarbituric acid. [7, 8, 9].

METHODS

1. The oxidation rate of the compounds resulting from the degradation of pectin and N-acetylneuraminic acid with the use of periodate, was examined on the basis of the method for determination of N-acetylneuraminic acid acc. to Skoza and Mohos [8].

40 cc of N-acetylneuraminic acid at concentration 100 μg/cc and 40 cc of diluted liquid after transelimination of pectin with pectate lyase, were introduced to separate 100 cc flasks; then 10 cc of 0.025 N ortoperiodate acid in 0.125 N sulphuric acid were added and incubated for 24 h at 288°K, 298°K and 308°K.

2.5 cc of liquid were sampled periodically from each of the flasks and added to dimethylformamide in order to preserve and intensify the colour of the solution. After cooling, the absorption of the solution at wavelength 549 nm was measured with the use of a Specol colorimeter.

2. The quantity of periodate used for oxidation of compounds resulting from the transelimination of pectin was determined by the method of Malaprate [3].

The mixture of unsaturated acids was obtained as a result of pectin transelimination with the use of pectate lyase obtained after cultivation of *Penicillium sp.* strain and after separation of pectin and enzymes on a semipermeable membrane through which only low molecular weight particles could pass.

The level of the products of pectin degradation was determined on the basis of the method given in items 4 and 5.

3. The analysis of absorption spectra of oxidized N-acetylneuraminic acid and acids resulting from pectin degradation after colour reaction with 2-thiobarbituric acid acc. to the method given in item 1, was made with the use of spectrocolorimeter Specol within the range of 500-600 nm wavelength.

4. Determination of calibration curve [8]. 0.1-0.7 μmol of N-acetylneuraminic acid was introduced into separate tubes supplemented with distilled water up to 2 cc, and 0.5 cc 0.025 N ortoperiodic acid in 0.125 N sulphuric acid were added and incubated for 30 minutes at 298°K. After incubation 0.5 cc of 2% sodium arsenine solution in 0.5 N hydrochloric acid and 1 cc solution of 2-thiobarbituric acid, previously neutralized to pH = 9 with the use of sodium hydroxide were added to each of the samples.

The mixture was heated in a boiling water bath for 15 minutes, then 6 cc of dimethylformamide were added and the absorption was measured at wavelength 549 nm with the use of a Specol spectrocolorimeter.

5. Determination of pectate lyase activity.

To 3 cc of 1% solution of pectin in Mc Ilvain buffer with pH = 5.8, 2 cc of liquid containing active enzymes of pectate lyases were intro-

duced; then it was incubated at 313°K for 1 h. After incubation 2 cc of reaction mixture were introduced to each of 2 tubes, 0.5 cc of 0.025 N periodate solution in 0.125 N sulphuric acid were added and incubated at 298°K for 2 hours. Then 0.5 cc of 2% sodium arsenide solution in 0.5 N hydrochloric acid were added and after 2 minutes, 1 cc of 1% 2-thiobarbituric acid, previously neutralized to pH = 9 with the use of sodium hydroxide, was added. The mixture was heated in a boiling water bath for 15 minutes, then 6 cc of dimethylformamide were added and absorption was measured at 549 nm. On the basis of the obtained measurements of the calibration curve, readings of the level of acids resulting from transelimination of pectin in the mixture, were made. Parallelly to the determinations, control measurements were performed, according to the generally accepted rules for this type of determination.

RESULTS

Among the colorimetric methods used for the determination of N-acetylneuraminic acid level, the colour reaction with 2-thiobarbituric acid is characterized by the highest molar coefficient of absorpition equal to 68 000 l/mol×cm (8).

As a result of oxidation of N-acetylneuraminic acid with the use of periodate in order to produce colour reaction with 2-thiobarbituric acid as well as a result of oxidation of 4-deoxy -5-ketouronic acid and unsaturated 4, 5-digalacturonic acid with the use of periodate, the same β-formyl-pyruvic acid giving a colour complex with 2-thiobarbituric acid, is produced.

Therefore, the possibility arose to utilize N-acetylneuraminic acid as a standard for plotting the standard curve in order to perform quantitative determinations of pectin transelimination products.

Fig. 1 and 2 illustrate dependences between the oxidation rate of N-acetylneuraminic acid and pectin degradation products which are mainly constituted by the above mentioned acids, periodate and temperature of reaction.

From the presented relationships it results that during the oxidation of N-acetylneuraminic acid, the maximal amount of β-formyl-pyruvic acid is accumulated after 30 minutes, irrespectively of the temperature of the process.

However, incubation of the mixture reacting at 308°K caused a gradual drop of the level of β-formyl-pyruvic acid after 1 h of oxidation.

The process of oxidation of pectin transelimination products was dependent in a significant way on the temperature of incubation of the reaction mixture what is illustrated in Fig. 2. The maximal concentration of β-formyl-pyruvic acid during the oxidation of pectin transelimination products at 288°K was obtained after 8 h incubation while at 298°K it

was reached after 2 h incubation and at 308°K — after 1 h. The incubation of reaction mixture at 308°K caused gradual lowering in absorption of the colour complex with thiobarbituric acid.

It should be, therefore, assumed that the most favourable conditions for the process are: 298°K for 2 hours.

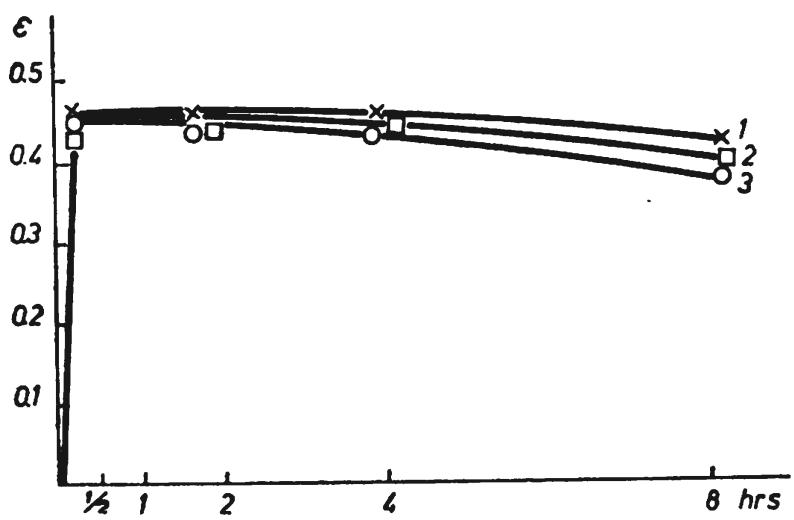

Fig. 1. Effect of temperatures on the oxidation velocity of N-acetylneuraminic acid by periodic acid, temperature: 1 — 288 K, 2 — 298 K, 3 — 308 K

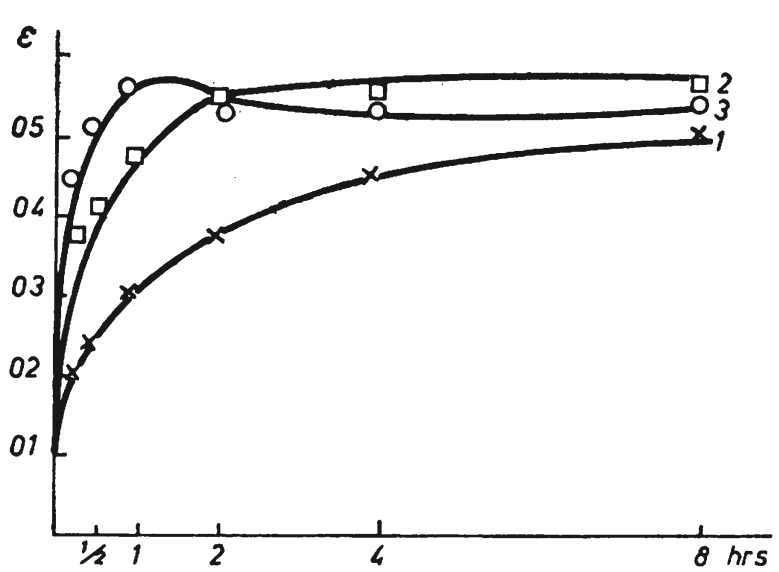

Fig. 2. Effect of temperature on the oxidation velocity of unsaturated galacturonic acid by periodic acid, temperature: 1 — 288 K, 2 — 298 K, 3 — 308 K

The determinations of the amount of periodate used for oxidation of pectin transelimination products show that 1 mole of these products determined acc. to the proposed method, needs 1.1-1.15 moles of periodate. Theoretically, for the oxidation of 1 mole of final products of pectin transelimination 1 mole of periodate is necessary; in practice however, due to the presence of other compounds in the reaction mixture undergoing slow oxidation [2], the amount of periodate used, is slightly higher than the theoretically calculated value.

The analysis of absorption spectra of colour complexes produced from 2-thiobarbituric acids and products of N-acetylneuraminic acid oxidation

and of pectin transelimination, points out to the identity of these spectra in the range of wavelength 500-600 nm with the absorption maximum 549 nm (Fig. 3).

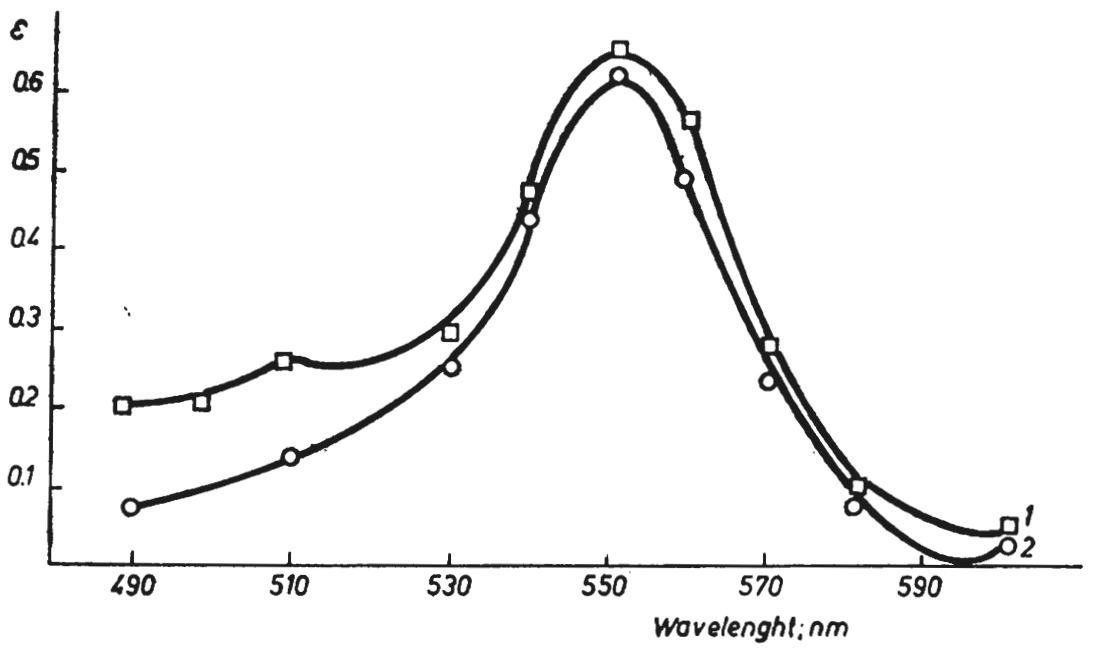

Fig. 3. Absorbance — wavelength plots for colour products in solution after oxidation; 1 — N-acetylneuraminic acid, 2 — unsaturated galacturonic acids (colour development with 2-thiobarbituric acid)

The above relationship show that as a result of oxidation of N-acetylneuraminic acid and of pectin transelimination products with the use of periodate, the same compound is produced, i.e. β-formyl-pyruvic acid and the reaction of oxidation of these substrates is of a quantitative and selective character.

Table. The statistic analysis of the results of the pectate lyase activity determination

Preparation	Amount of repeat.	P L activity μmol/min × 10^{-3}	Mean square deviation	Standard deviation	Variation coeffic
1	8	55.4±2.7	27.1	1.97	3.56
2	10	74.7±4.3	55.1	2.47	3.3
3	7	102.4±7.1	90	3.87	3.7

The data presented in Table and concerning the statistical evaluation of the suggested method for a determination of pectate lyases, indicate that the proposed method is characterized by considerable accuracy and small dispersion of the results (coefficient of variability 3.52%).

DISCUSSION

The ability of pectate lyases to degrade pectines depend on the degree of pectin esterification, length of the chain, structural differences and other

factors [12]. In spite of this, there is a tendency to determine the activity of pectate lyases in absolute units.

The determination of the activity of pectate lyases on the basis of measurements of changes in light absorption at wavelength equal 235 nm, is characterized by low sensitivity what is evidenced by molar absorption = 4600 l/mol×cm. The method gives lowered results because of a lack of absorption maximum at 235 nm [8] in the spectrum of 4-deoxy-5-ketouronic acid being the final product of pectin transelimination.

According to Weissbach and Hurwitz [11] the oxidation with periodate of 2-keto 3-deoxyacids to which belong 4-deoxy-5-ketouronic acid and N-acetylneuraminic acid, results in the formation of β-formyl-pyruvic acid which produces a colour complex with thiobarbituric acid and in case of N-acetylneuraminic acid, the molar absorption is 68 000 l/mol×cm what is a proof of the high sensitivity of colour reaction. From the experiments of Skoza and Mohas [9] it results that the method of determination of N-acetylneuraminic acid is more suitable than the previously applied methods [1, 10] because it is less labour consuming and more accurate.

The utilization of the method for the determination of pectin transelimination products required additional studies connected with the quantitative determination of the oxidation process of the compounds examined with the use of periodate. The results of studies on the rate of oxidation of N-acetylneuraminic acid show that the time of oxidation should be 30 minutes at 298°K and complete oxidation of pectin transelimination products into β-formyl-pyruvic acid at 298°K is obtained after 2 h.

Specifity of the reaction of oxidation of the pectin degradation products in conditions of determination and suitability of N-acetylneuraminic acid as a standard is confirmed by the results of consumption of 1.1-1.15 moles of periodate for oxidation of 1 mole of pectin transelimination products, the concentration of which was calculated on the basis of a standard curve plotted for N-acetylneuraminic acid.

Bearing in mind the following dependences as well as the identity of absorption spectra within the range of 500-600 nm wavelength for products resulting from oxidation of N-acetylneuraminic acid and pectin degradation products, it may be stated that N-acetylneuraminic acid is a suitable standard for colorimetric determination of acids resulting from pectin transelimination and the method of its determination is suitable for a quantitative measurement of the concentration of these acids.

CONCLUSIONS

1. The method elaborated for determination of activity of pectate lyases with the use of N-acetylneuraminic acid as a standard in colori-

metric determination of enzymatic products of pectin transelimination with the use of 2-thiobarbituric acid allows to express the enzymatic activity in the absolute units.

2. On the basis of literature and of our own experiments it may be stated that the suggested method for determination of pectate lyases activity is superior to the method utilizing the increase of light absorption at 235 nm during the enzymatic transelimination of pectin both in terms of sensitivity and identity of the reaction products (4-deoxy-5-ketouronic acid).

3. The disadvantage of the recommended method for determination of pectate lyases activity is that the determinations are time consuming, therefore, it is necessary to conduct further studies on the modification of the method in order to shorten the time of the analyses.

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Authors address: 20-934 Lublin, Akademicka 13

Z. *Targoński*

KWAS N-ACETYLONEURAMINOWY JAKO WZORZEC PRZY OZNACZANIU AKTYWNOŚCI LIAZ PEKTYNOWYCH Z KWASEM TIOBARBITUROWYM

Instytut Chemii i Technologii Rolnej, Akademia Rolnicza, Lublin

Streszczenie

Opracowano kolorymetryczną metodę oznaczania aktywności liaz pektynowych w jednostkach międzynarodowych, wykorzystując reakcję barwną kwasu 2-tiobarbi-

turowego z kwasem β-formylopirogronowym powstałym w wyniku utleniania produktów transeliminacji pektyny nadjodanem.

Jako substancję wzorcową wykorzystano kwas N-acetyloneuraminowy, który w wyniku utleniania nadjodanem przechodzi również w kwas β-formylopirogronowy. Za optymalną temperaturę utleniania przyjęto 298°K, zaś optymalny czas utleniania nadjodanem kwasu N-acetyloneuraminowego wynosi 30 min, a dla produktów transeliminacji pektyny 2 h.

Stwierdzono identyczność widm absorpcyjnych kompleksu barwnego kwasu 2-tiobarbiturowego i produktu utleniania nadjodanem kwasu N-acetyloneuraminowego oraz kwasów powstałych w wyniku transeliminacji pektyny w zakresie długości fal 500-600 nm z maksimum absorpcji przy 549 nm. Proponowana metoda oznaczenia aktywności liaz pektynowych charakteryzuje się znaczną dokładnością i niewielkim rozrzutem wyników, o czym świadczy niski współczynnik zmienności równy 3,5%.

Omówiono zalety powyższej metody w porównaniu z metodą wykorzystującą zmiany absorpcji światła podczas transeliminacji pektyny przy długości fali 235 nm.